Kidney International, Vol. 67 (2005), pp. 1286–1296

Evidence for low-density lipoprotein–induced expression of connective tissue growth factor in mesangial cells

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Background. Although hyperlipidemia is a risk factor for the progression of renal damage, the relationship between increased plasma lipoproteins and glomerular injury is poorly defined. Connective tissue growth factor (CTGF) is emerging as a key determinant of progressive fibrotic diseases and its expression is up-regulated by diabetes. To define the mechanisms through which low-density lipoproteins (LDLs) promote glomerular injury, we evaluated whether LDL can modulate the expression of CTGF and collagen I.

Methods. The effects of LDL on CTGF and collagen I expression were carried out in rat mesangial cells.

Results. Treatment of mesangial cells with LDL for 24 hours produced a significant increase in the protein levels of CTGF and collagen I compared to unstimulated controls. To explore if CTGF and collagen I are downstream targets for regulation by transforming growth factor-β (TGF-β), mesangial cells were treated with various concentration of TGF-β for 24 hours. TGF-β produced a concentration-dependent increase in the protein levels of CTGF and collagen I. The increase in CTGF and collagen I induced by LDL was significantly inhibited by neutralizing anti-TGF-β antibodies. Inhibition of $p38^{mapk}$ or $p42/44^{mapk}$ activities did not affect LDL-induced TGF-β1, CTGF, and collagen I expression, whereas inhibition of c-Jun NH2-terminal kinase (JNK) suppressed LDL-induced TGF-β, CTGF, and collagen I expression.

Conclusion. These findings implicate JNK pathway and TGF-β1 as key players in LDL signaling leading to CTGF and collagen I expression in mesangial cells. The data also point to a potential mechanistic pathway through which lipoproteins may promote glomerular injury.

Diabetic nephropathy is the leading cause of end-stage renal failure, and is clinically manifested by albuminuria, hypertension, and a progressive decline in glomerular filtration rate (GFR) [1–3]. About 30% to 40% of patients with type 1 diabetes develop progressive nephropathy [3]. A very characteristic and initial event of the development of diabetic nephropathy is glomerulosclerosis, which is featured by increased thickness of the glomerular basement membrane (GBM), and a widening of the mesangium with accumulation of extracellular matrix (ECM). The development of glomerulosclerosis is clearly dependent on hyperglycemia since intensive control of glycemia in type 1 diabetic patients was associated with a reduction of glomerular lesions [4]. Although the association of chronic hyperglycemia and diabetic nephropathy is well established, the risk factors and cellular signaling mechanisms that promote glomerulosclerosis in diabetes are still undefined. In this regard, the cytokine transforming growth factor-β (TGF-β) has been shown to play a pivotal role in mesangial cell expansion and matrix deposition [5]. Recent evidence indicates that the profibrotic signals initiated by TGF-β are mediated via activation of connective tissue growth factor (CTGF) [6].

CTGF was originally identified as a product of human umbilical vein endothelial cells that was both chemotatic and mitogenic for fibroblasts [7]. It is now known that CTGF belongs to a new gene family, CCN (named after prototype members of this family CTGF, cyr61, and nov) [8]. The molecular weight of CTGF-like factors varies between 35 and 40 kD, and the structure of these molecules consists of four modules: an N-terminal insulin-like growth factor binding protein (IGFBP)-like domain, a von Willebrand factor type C repeat domain, a thrombospondin type 1 repeat domain, and a C-terminal dimerization domain [8].

The biologic actions of CTGF are pleiotropic and seem to be cell specific, but the cellular mechanisms of its actions are still undefined. An emerging role of CTGF is that of a prosclerotic factor. Renal expression of CTGF is up-regulated by the diabetic state and by other progressive renal diseases [9]. In addition, CTGF has been shown to increase collagen I expression in human mesangial cells [10].

Poorly controlled type 1 diabetes is usually associated with elevated levels of plasma LDL, intermediate-density lipoproteins and very low-density lipoprotein levels

Key words: MAPK, TGF-β, collagen I, JNK.

Received for publication August 27, 2004
and in revised form October 1, 2004
Accepted for publication October 12, 2004

[11–14]. Besides quantitative abnormalities, patients with type 1 diabetes are known to have significant qualitative lipoprotein abnormalities. Abnormalities in lipid and lipoprotein metabolism are commonly associated with end-stage renal disease (ESRD) [15–17]. Specifically, hyperlipidemia and the glomerular deposition of atherogenic lipoproteins (LDL and oxidized LDL) are implicated in key pathologic processes involved in the development of glomerular disease, including stimulation of monocyte infiltration into the mesangial space, mesangial cell hypercellularity, and ECM deposition [17, 18].

Although hyperlipidemia is now considered a risk factor for the progression of renal damage, the relationship between increased plasma lipoproteins and glomerular injury is poorly defined. Hyperlipidemia can directly or indirectly stimulate the synthesis and release of factors from resident renal cells which in turn can stimulate mesangial cell growth, as well as ECM production in an autocrine or paracrine manner [19, 20]. Therefore, the present study was designed to explore the potential role of LDL in modulating the expression of CTGF in mesangial cells and to delineate the cellular signaling mechanisms through which this regulation may occur.

METHODS

Mesangial cell culture

Rat glomerular mesangial cells were prepared by a modification of the method of Lovett et al [21]. Glomerular cells collected as described above were incubated in phosphate-buffered saline (PBS) plus 0.1% gentamysin solution and 1% antibiotic antimycotic, pH 7.4, containing collagenase (5mg/mL), at 37°C for half an hour to remove epithelial cells, leaving the glomerular cores containing mesangial and endothelial cells, vortexed every 10 minutes during the incubation. The cores were diluted in 1.5 mL RPMI 1640 medium with Hepes and L-glutamine (Invitrogen Corporation, Carlsbad, CA, USA) per kidney, containing 0.1% gentamycin solution, 1% antibiotic antimycotic, 0.5% insulin transferring solution, and 20% fetal bovine serum (FBS), conditions which favor growth of mesangial cells. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5%CO_2. Cell viability was assessed by standard dye exclusion techniques, using 0.1% Trypan blue. Mesangial cells were identified by the following criteria. Mesangial cells stained positive for intracellular cytoskeletal fibrils of actin and smooth muscle cell (SMC)-specific myosin (indicative of contractile cells), desmin, and vimentin and negative for cytokeratin and factor VIII antigens. Morphologically, mesangial cell had an elongated and stellate or spindle-shaped morphology. Mesangial cells isolated by this procedure were homogenous and used in all studies between passages 3 and 8.

LDL preparation and characterization

LDL was prepared as previously described [22]. Briefly, blood was taken from fasting healthy nondiabetic volunteers into a lipoprotein preservative/antioxidant cocktail (LPPC) containing ethylenediaminetetraacetic acid (EDTA) (0.1% wt/vol), chloramphenicol (20 μg/mL), gentamycin sulfate (50 μg/mL), and ε-amino-n-caproic acid (0.13%, wt/vol). Phenylmethylsulfonyl fluoride (PMSF), 20μg/mL final concentration, was added to plasma to retard proteolysis. All samples were processed at low temperature and in the absence of white light to minimize oxidation. All density solutions were supplemented with LPPC, degassed, and purged with N_2. Plasma density was increased to d = 1.21 g/mL using dried KBr and 11 mL layered under d = 1.019 g/mL saline/LPPC. After ultracentrifugation (Beckman VTi50 rotor), $2\frac{1}{2}$ hours 50,000 rpm 7°C with slow acceleration and deceleration, the LDL band was harvested by piercing the tube and aspirating into a syringe. LDL isolated by this procedure was free from contamination with apolipoprotein A (ApoA-I) and albumin.

Each LDL preparation was characterized for purity by electrophoresis on 1% agarose gels (Paragon gels) (Beckman, Brea, CA, USA). The LDL pools were tested for endotoxin contamination by the Limulus Amebocyte Lysate (BioWhittaker, Walkersville, MD, USA) according to the manufacturer's suggestion.

Cell extracts

Mesangial cells treated with LDL were washed twice in ice-cold PBS, scraped in PBS containing 2 mmol/L sodium vanadate, and centrifuged at 3000*g* for 5 minutes. Pellets were resuspended in 100 mL of lysis buffer (20 mmol/L Tris, 130 mmol/L NaCl, 10% glycerol, 10 mmol/L CHAPS, 1 mmol/L PMSF, 2 mmol/L sodium vanadate, 100 mU/mL aprotinin, and 0.156 mg/mL benzamidin, pH 8.0), incubated on ice for 30 minutes and centrifuged at maximum speed for 5 minutes. The supernatant was used as the protein source and its concentration was determined by a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard protein.

Western blotting of CTGF and collagen I

Mesangial cells were cultured in 6-well plates (9.6 cm^2/well). At 80% confluence, cells were serum starved by the changing of serum-free media (RPMI medium1640 with 25 mmol/L Hepes buffer, 11.11 mmol/L glucose, and L-glutamine) within 24 hours. Quiescent mesangial cells were stimulated with LDL (50 μg/mL) for 24 hours in the presence and absence of either a p42/$p44^{mapk}$ inhibitor (PD98059), 40μmol/L (Calbiochem, La Jolla, CA, USA), a $p38^{mapk}$ inhibitor (SB203580), 10 μmol/L (Calbiochem), and/or the c-Jun NH2-terminal kinase (JNK)